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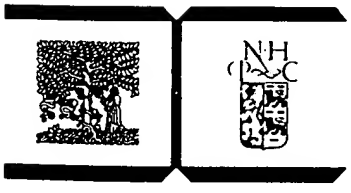
Proceedings of the Symposium on Plasmids of Medical, Environmental and Commercial Importance held in Spitzingsee, F.R.G., 26-28 April, 1979.

Editors
K.N. TIMMIS
and
A. PÜHLER



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RESEARCH ARTICLES

I – PLASMIDS OF MEDICAL IMPORTANCE: GENES AND PRODUCTS

Plasmids of Medical, Environmental and Commercial Importance
K.N. Timmis and A. Pühler, editors

THE CHARACTERIZATION OF AN ESCHERICHIA COLI PLASMID DETERMINANT THAT ENCODES FOR THE PRODUCTION OF A HEAT-LABILE ENTEROTOXIN

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INTRODUCTION

Certain strains of Escherichia coli are the etiologic agents for non-invasive gastroenteritis in man and young animals[1,2]. The disease is cholera-like in nature and the causative agents have been shown to elaborate one or two enterotoxins[3]. These E. coli also produce an adhesin that enables them to colonize the upper small bowel[4], a site not normally inhabited by E. coli[5]. In many of these enterotoxigenic E. coli, the genetic determinants for toxigenicity and adherence have been shown to be part of plasmids[6]. Smith et al.[6] used the transmissible nature of these plasmids to study the contribution of each virulence factor to the pathogenesis of the disease in piglets. They concluded that both virulence factors were necessary for the manifestation of the disease. They also reported that the introduction of the two virulence factors into wild-type E. coli sometimes created a fully pathogenic strain.

Enterotoxigenic E. coli have been shown to make two distinct enterotoxins, ST and LT[3]. These toxins are discernable by several characteristics including biological activity, immunogenicity, and relative heat stability[2]. Burgess et al.[7] have reported that there are two distinct ST's that can be discerned by biological assays. STa elicits fluid accumulation in suckling mice while STb is only active in weaned piglets and rabbit ligated loops. Alderete et al.[8] have reported STa to be a low molecular mass protein (5,100 daltons). LT is a larger protein[2] that shares several characteristics with the toxin made by Vibrio cholerae. Both toxins have been shown to have an ADP-ribosylating activity (M. Gill personal communication) and they both stimulate adenyl cyclase in eukaryotic cells[9,10]. LT and cholera toxin also show partial immunological cross-reactivity and have similar membrane receptors[11,12].

Smith et al.[13] first used the term Ent to describe enterotoxin encoding plasmids. Ent plasmids can encode LT only, ST only, and both LT and ST[2]. So et al.[14] showed that LT + ST plasmids constituted a homogeneous group of extrachromosomal elements that had similar mole fraction guanine + cytosine, molecular mass (about 60 Mdal), and DNA sequence homology. On the other hand, ST plasmids were found to constitute a disparate group of plasmids. Following

the successful isolation of the ST genetic determinant in our laboratory[15], So et al.[16] reported that the ST genetic determinant was part of a transposon that was flanked by inverted and repeated ISl elements.

We have been interested in exploring the relatedness of the LT gene(s) from enterotoxigenic E. coli strains isolated from humans and animals. In our work we studied an LT gene(s) that was cloned from an Ent plasmid isolated from an E. coli strain pathogenic for piglets[17]. During the course of our work, we determined that LT was a multimeric toxin. The 25,500 dalton subunit was shown to have an adenyl cyclase stimulating activity and the 11,500 dalton subunit an adsorption activity. The cistrons that encoded these proteins were located on a genetic map of the LT DNA region. We used, as a hybridization probe, a DNA fragment that contained all of the 11,500 dalton protein cistron and approximately 10% of the 25,500 dalton protein cistron to study the sequence homology among LT genes of different origins. Our results indicated that LT genes have similar structures but they are not identical.

MATERIALS AND METHODS

Bacterial strains and plasmids. The E. coli sublines used in this study have been described in detail previously[17,18]. The plasmids have also been described[17,19].

Recombinant DNA methods. Agarose gel electrophoresis, restriction endonuclease cleavage reactions, and polynucleotide ligase conditions have been described by So et al.[17]. The experiments reported here were performed under P2-EKl conditions as specified in the NIH Guidelines for Recombinant DNA Research.

Protein synthesis in minicells. E. coli minicells were isolated from E. coli DS410 and the plasmid-specified proteins were labelled as described by Dougan et al.[18]. Labelled minicells were analyzed by the electrophoretic method of Studier[20]. Staphylococcus-antibiody-antigen reaction conditions have been described previously[21].

Adenyl cyclase assays. Adenyl cyclase stimulating activity was determined by D. M. Gill using a method that has been described[9].

Hybridization reactions. DNA was labelled by nick translation as described by Rigby et al.[22]. Colony filter hybridizations were performed as described by Grunstein[23]. Filter blot hybridization reactions were performed as described by Southern[24] and modified by Botchan et al.[25].

RESULTS

The LT genetic determinant was initially isolated on a 5.8 MdaI DNA fragment[17] and was subsequently joined to the carrier plasmid, pBR313. Normally the LT gene(s) is found in enterotoxigenic E. coli in one or two copies per chromosome equivalent. The cloned LT DNA fragment was found to be present at approximately 20 copies per chromosome equivalent. Laboratory E. coli strains harboring the cloned toxin gene(s) were found to be hypertoxin producers presumably as a result of gene amplification. A more precise location of the LT genetic determinant within the cloned DNA was established by introducing deletions into the plasmid. Specific DNA fragments were deleted from the plasmid by making partial plasmid digests with a restriction enzyme followed by rejoining the DNA fragments with ligase (Figure 1). We were able to isolate

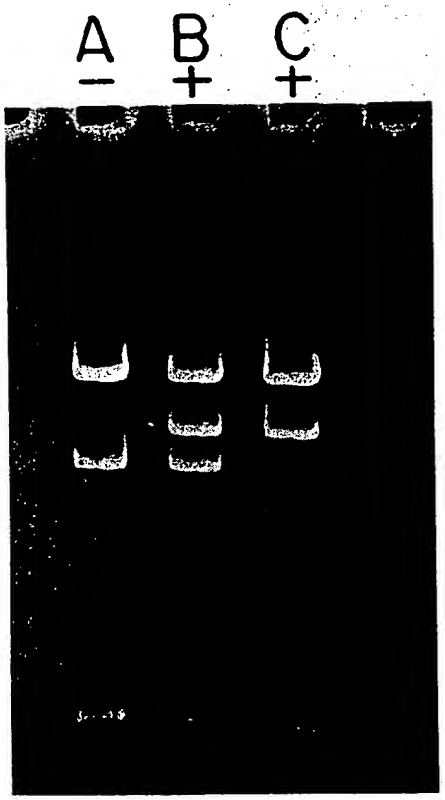

Fig. 1. HindIII cleavage of EWD022 and HindIII generated deletion plasmids. Plasmid DNA was purified, cut with HindIII, and electrophoresed through a vertical 1% agarose gel: (A) EWD306, (B) EWD022, (C) EWD300. The + or - indicates if the plasmid encoded functional LT as measured in the Y-1 adrenal

cell tissue culture assay[26]. Both EWD300 and EWD306 were derived from EWD022 by deleting a HindIII DNA fragment.

deletions which spanned the entire cloned DNA region. The effect of each deletion on LT expression was determined by using a tissue culture assay for LT[26]. In this system, Y-1 adrenal cells change shape from cubic to round in the presence of a functional LT. Using this type of analysis, we identified a 1.2 Mdal region that contained the LT genetic determinant (Figure 2). Deletions

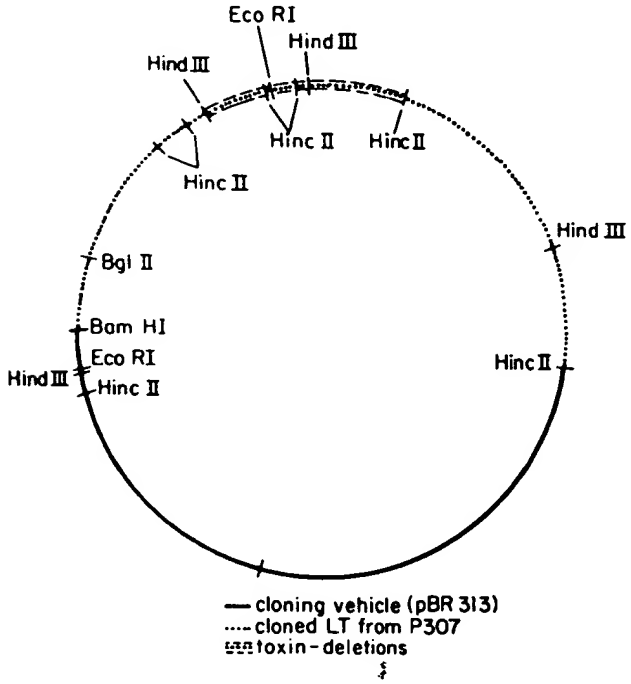

Fig. 2. Schematic diagram of restriction enzyme recognition sites in EWD022. Deletions that extended into the region bounded by the HindIII and HincII sites resulted in the loss of the LT^+ phenotype.

in this region resulted in the loss of the LT^+ phenotype. The 1.2 Mdal region had a coding capacity for about 600 amino acids. This can be considered as an upper size limit for an LT of single subunit composition.

The protein products encoded by the LT DNA region were identified using minicells as an in vivo protein synthesizing system[27]. EWD299 is a deletion plasmid that contained only the 1.2 Mdal LT DNA region. Analysis of this plasmid in minicells revealed that twelve proteins were encoded by the plasmid (Figure 3). The LT proteins were identified in minicell extracts by using an anti-serum prepared against a crude LT preparation. Since the amount of protein

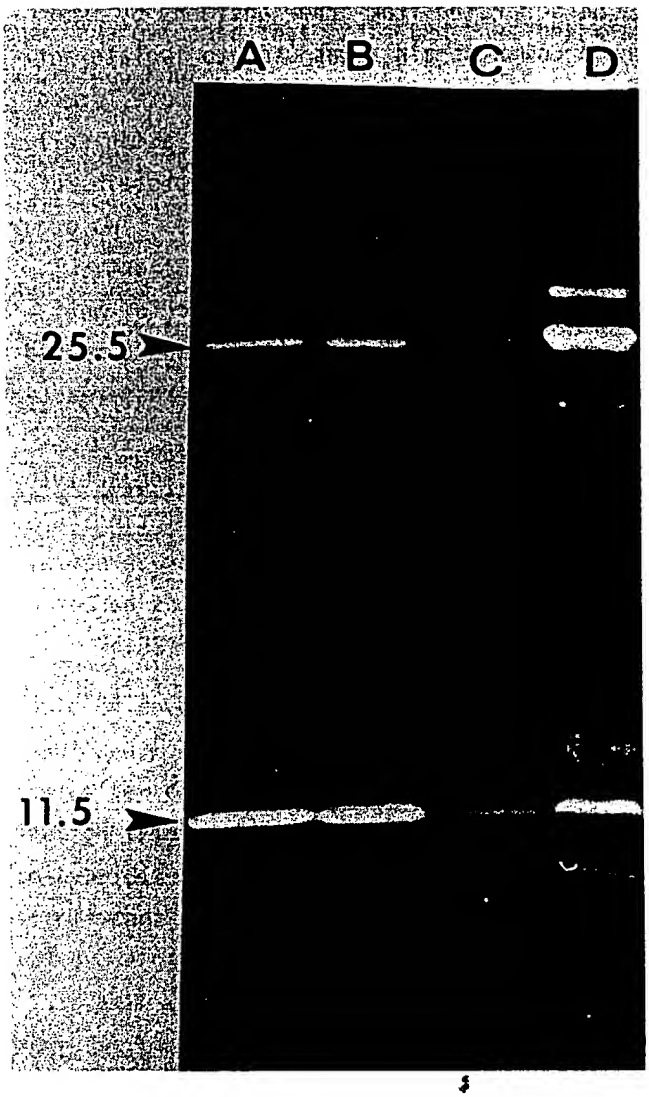

Fig. 3. Autoradiograph of a polyacrylamide gel containing total EWD299 encoded proteins and proteins that react with anti-sera. A cytoplasmic-periplasmic fraction from minicells containing EWD299 was mixed with anti-sera and formalized S. aureus. The cells were pelleted through 1M sucrose, boiled in final sample buffer, and electrophoresed through a 10-15% linear gradient SDS-polyacrylamide gel: (A) anti-cholera toxin serum, (B) anti-cholera toxin subunit B serum, (C) anti-LT serum, (D) total EWD299 encoded proteins. Two proteins, 25.5K and 11.5K, were found to react specifically with all three anti-sera.

made in minicells is too small to be separated from other antigens by immunoprecipitation, Staphylococcus aureus Cowan strain I was used to isolate antigen-antibody complexes[28]. Tertiary complexes of S. aureus-Ab-Ag are formed and these aggregates can be separated from unreacted antigens in a minicell lysate by pelleting the reaction mixture through 1M sucrose. A protein 26K in mass was found to bind non-specifically to the S. aureus cells. Two other proteins 25.5K and 11.5K in mass were complexed in the presence of anti-LT serum. Both of these proteins were also complexed in the presence of anti-cholera toxin subunit B serum. These results indicated that LT was composed of two distinct subunits, at least one of which immunologically cross-reacted with cholera toxin subunit B.

LT has been shown to have two separate activities: an adsorption activity for certain eukaryotic cell membranes and an adenyl cyclase stimulating (ACS) activity. ACS activity can be measured independently in pigeon erythrocyte lysates (PEL)[10]. Most of the ACS activity in extracts from strains harboring EWD299 migrated in SDS-polyacrylamide gels at a molecular mass of 25.5K (Figure 4). A smaller peak of activity was identified at a mass of 21.5K. We

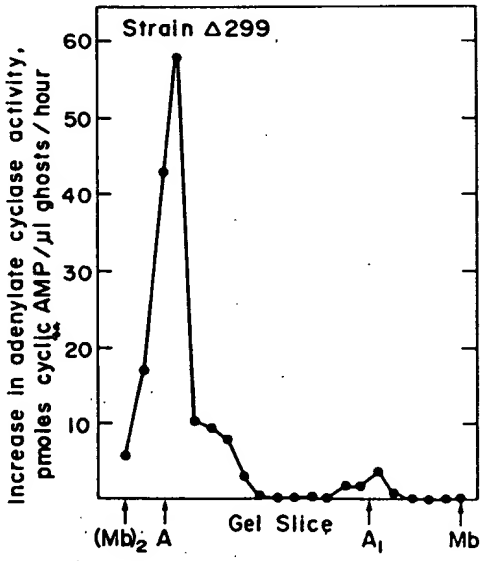

Fig. 4. Adenyl cyclase stimulating (ACS) activity encoded by EWD299. Extracts were prepared from strains carrying EWD299 and fractionated by 10-15% linear SDS-gradient PAGE. The gels were sliced into fractions, the proteins in each gel slice were eluted, and the preparations were assayed for ACS activity. A and A_1 indicate the migration distance of cholera toxin subunit A and A_1, respectively[29]. Mb is the monomer form of myoglobin and Mb_2 is the dimer form. This experiment was performed in collaboration with D.M. Gill and a more complete description of this collaborative work will appear elsewhere.

now have evidence that indicated that the smaller molecular mass activity was a breakdown product of the 25.5K protein (data not shown). The tissue culture assay for LT required both the adsorption activity and the ACS activity. Therefore, strains that had ACS activity but were not positive in tissue culture would be deficient in adsorption activity. Analysis of the deletion plasmids revealed that the 11.5K protein perfectly correlated with adsorption activity. These results indicated that LT was a multimeric toxin composed of a 25.5K protein that had ACS activity and an 11.5K protein that had an adsorption activity. We also have evidence which indicated that the holotoxin was composed of one 25.5K subunit and five 11.5K subunits.

We have been able to locate the relative positions of the two LT cistrons on a genetic map of the LT DNA region (data not shown). A 0.5 Mdal HindIII DNA fragment was identified and shown to encompass all of the 11.5K protein cistron and approximately 10% of the 25.5K protein cistron. This DNA fragment was used as a nucleic acid hybridization probe to determine the relatedness of LT genes of diverse origins. Initially, we wanted to determine if Ent plasmids could be detected by the colony filter blot hybridization technique[23]. Our results demonstrated that LT genes present at a concentration of one or two copies per chromosomal equivalent could be detected by colony filter hybridization (Figure 5). Furthermore, all LT^+ E. coli isolates tested were found to hybridize to the probe. These preliminary data indicated that LT genes share significant sequence homology. The colony hybridization method may prove useful for studying the epidemiology of LT enterotoxigenic E. coli.

To more precisely determine the degree of homology among LT genes, we chose four Ent plasmids to study in detail. Two of the plasmids were isolated from E. coli pathogenic for humans and the other two were isolated from porcine strains. One of the porcine Ent plasmids was P307, the plasmid from which the hybridization probe was isolated. The other porcine Ent plasmid was pCG86[30]. Plasmid DNA was purified from each strain, cut with HindIII, and hybridized to the HindIII probe by the method of Southern[24]. Only one DNA fragment in each of the four samples hybridized to the probe and in all cases the fragment was 0.5 Mdal in size (data not shown). This result indicated that most, if not all, of the HindIII DNA portion of the LT DNA region was conserved among the Ent plasmids. We performed the same type of analysis using six different restriction enzymes. In this way, we studied the internal structure of the common HindIII fragment. For example, the HindIII probe was found to contain a single SmaI site. Therefore, cleavage of P307 with SmaI (followed by blotting and hybridization) resulted in the appearance of two DNA bands that hybridized to the probe (Figure 6). Using enzymes that cleaved within the hybridization

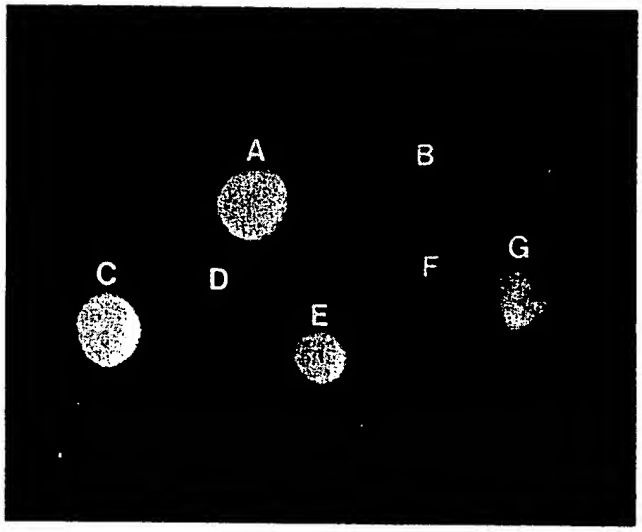

Fig. 5. Colony filter hybridization of LT^+ and LT^- E. coli. The 0.5 Mdal HindIII fragment was isolated and labelled with ^{32}P by nick translation and hybridized to colonies that were lysed on nitrocellulose filters: (A) P307, the Ent plasmid from which the LT genetic determinant was cloned; (B) C600, an LT^- laboratory strain; (C) H10407, an LT^+ strain pathogenic for humans; (D) a spontaneous LT^- derivative of H10407; (E), (F), (G) LT^+ strains pathogenic for humans.

probe, we could determine if these sites were conserved in all the Ent plasmids. Similarly by using enzymes that did not cleave within the hybridization probe, we could determine if new restriction enzyme sites were present in the LT DNA region of the Ent plasmids (Figure 6). From this analysis, we determined that the human Ent plasmids apparently demonstrated a small degree of structural divergence when compared to each other and to the hybridization probe. One human Ent plasmid was found not to have an SmaI site within the common HindIII fragment (Figure 6) and the other human Ent plasmid did not have an EcoRI site within the common HindIII fragment (data not shown). However, an enzyme that produced two DNA fragments of equal size both of which hybridized to the probe would appear as a single fragment in this type of analysis. Initially, the two porcine Ent plasmids appeared to have LT genetic determinants with very different structures. With five of the enzymes, pCG86 was found to have one extra DNA fragment that hybridized to the probe when compared to P307. However, all but the "extra" DNA fragment were found to be the same molecular mass as the corresponding restricted P307 DNA fragment(s). This unexpected observation

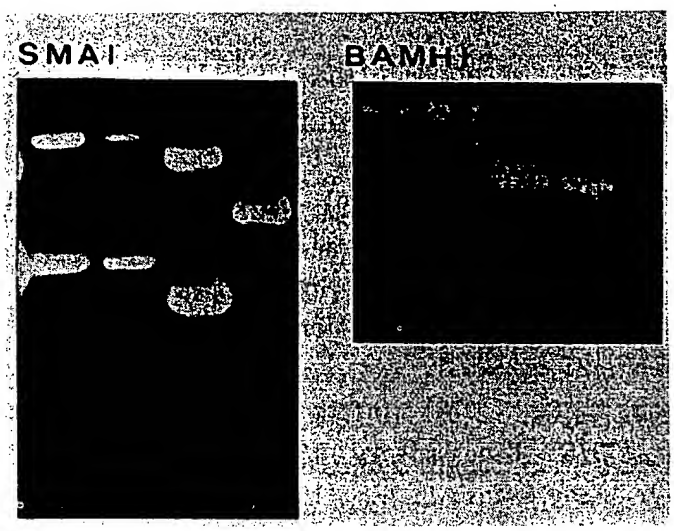

Fig. 6. Autoradiographs of Southern blots of restricted Ent plasmids. The DNA in the first autoradiograph was cut with SmaI. The order of the samples is (1) P307, (2) pCG86, (3) and (4) human Ent plasmids. The DNA in the second autoradiograph was cut with BamHI. The order of the samples is (1) and (2) human Ent plasmids, (3) pCG86, (4) P307.

can best be accomodated by a model in which all or part of the LT DNA region is duplicated in pCG86. We have other evidence that the LT DNA duplication probably occurs as an inverted and repeated sequence. Confirmation of this hypothesis awaits heteroduplex analysis. The similarity or divergence of the sequences adjacent to the LT DNA region is indicated by the sizes of DNA fragments that hybridized to the probe. A comparison of the sizes of the hybridized fragments of P307 and pCG86 indicated that the DNA sequences adjacent to the LT DNA region in each of these plasmids are the same (Figure 6). In contrast, the human Ent plasmids differed markedly from each other and from the porcine Ent plasmids with respect to the sequences adjacent to the LT DNA region. By analyzing more human and porcine Ent plasmids, we will be able to determine if indeed the porcine Ent plasmids constitute a homogeneous group and the human Ent plasmids a heterogeneous group as the preliminary data indicated.

Acknowledgements

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